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THE PHYSIOLOGICAL RESPONSE OF THE ESTUARINE CLAM, *RANGIA CUNEATA* (GRAY), TO SALINITY. II. UPTAKE OF GLYCINE [1]

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The literature concerning the uptake and utilization of dissolved organics by estuarine and marine invertebrates has been reviewed by Stephens (1972). Stephens (1964) reported that the capacity of two polychaetous annelids, *Nereis limnicola* and *N. succinea* to take up C^{14}-labeled glycine was related to the chloride concentration of the acclimation and exposure media. For both species, the uptake was nearly zero at chlorosities of 150 meq Cl^-/l (10‰ S) or less, and a significant increase in glycine removal occurred at 200 meq (about 13‰). From the work of Smith (1959) and the data of Oglesby (1965), it appears that these polychaetes begin chloride regulation at approximately 210–225 meq (14‰ S).

Stephens and Virkar (1966) studied the uptake of glycine and other amino acids by the brittle star, *Ophiactis arenosa*. Since these animals are not truly euryhaline, they were only tested over a salinity range of 50 to 100% sea water (16.5–33.0‰). Post-incubation after exposure to C^{14}-glycine showed a decrease in salinity was correlated with a decrease in ninhydrin positive material (NPM), and an increase in the radioactivity associated with the alcohol insoluble fraction of the animals. There was a corresponding decrease in the alcohol soluble radioactivity as salinity decreased from 70 to 50% sea water.

The literature regarding the utilization of free amino acids (FAA) in the osmoregulation of lamellibranch mollusks and other estuarine and marine invertebrates was reviewed by Virkar and Webb (1970). Gilles (1972) has shown that amino acids played a part in the cellular osmoregulation process in the intertidal bivalves studied. Allen (1961) found that *Rangia cuneata* taken from different salinities contained quantities of alanine, aspartic acid, glutamic acid and glycine, which increased as the salinity increased from 3 to 17‰. As the environmental

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salinity increased from 17 to 25‰ there was a slight decrease in the levels of these compounds. Alanine was found to be the most prominent amino acid in *Rangia,* while in *Mytilus edulis* taurine is retained in high concentration (Allen and Awapara, 1960).

The estuarine clam, *Rangia cuneata,* is extremely tolerant to wide salinity fluctuations (Hopkins and Andrews, 1970) and is of potential economic importance (Hopkins, 1970). Unusual among the bivalves which have been studied (Robertson 1964; Pierce, 1970), *Rangia* have been shown to be capable of osmoregulation in low salinity water (Bedford and Anderson, 1972). The blood osmotic concentration becomes hyperosmotic to the environment at salinities less than 10‰. From 5‰ to 1‰ salinity (S) an osmotic differential of approximately 55 to 65 milliosmoles per liter above the environment was maintained by the blood.

Since the osmoregulartory activity of *Rangia* had been established and since these clams were tolerant to salinity extremes, it was felt that they would be excellent subjects for study on the relationship between salinity and the uptake of amino acids from solution.

Methods

The specimens of *Rangia* were collected from McCollum Park, Trinity Bay, Chambers County, Texas. The salinity of this region fluctuates widely, but is generally between 5 and 15‰ S. The substrate was a very uniform hard sandy mud, and the water depth ranged between 45 cm at shore to 95 cm approximately 300 meters from shore. The density of the clam population at this site was between 45 and 49 individuals per square meter.

The animals were maintained in the laboratory in 15 gallon aerated aquaria containing water of the same salinity as that of the collection site. Water used for holding and experimental work was prepared with distilled water and Instant Ocean (Aquarium Systems, Inc.). After two days of maintenance at the collection salinity, they were acclimated in steps of 5‰ per 2 days. This procedure resulted in a survival rate of 98 per cent or greater. Animals were held at the final experimental salinity for at least 7 days, and no longer than 24 days before use. In every case all clams utilized in a given experiment were collected on the same date. There was no attempt to feed the animals, since preliminary studies have shown that they survive for over two months in the laboratory without food. Allen (1959) has reported that these animals survive long periods of starvation and no appreciable changes occur in the amino acid composition after 21 days without food. Maintenance and experimentation were in an air conditioned laboratory at 22 ± 1° C.

Per cent body water and per cent ash

After acclimation for 10 days at salinities from 1 to 32‰, clams were shucked, blotted, and the wet weight was taken. The tissue was dried to a constant weight (approximately 72 hours) at 95° C, and then ashed in a muffle furnace at 550° C to a constant weight (24 hours). These data were used to prepare curves for per cent body water and per cent ash-free dry weight at each salinity. Where direct determinations of ash-free dry weight were not possible in a given experiment, values were corrected using the above curves. To determine the rate at

which volume regulation occurred, clams were transfered from 15‰ to 5 or 25‰ S and analyzed for per cent water at various time intervals.

Glycine uptake

Animals utilized in uptake experiments were carefully selected for uniform size (55 ± 3 mm) to reduce variation due to weight. A random selection of 44 clams within this size range gave a mean ash-free dry weight of 1.31 grams and a standard deviation (SD) of 0.27 grams.

Whole animals were tested for their ability to remove C^{14}-labeled glycine at various salinities by exposing them for different time intervals to mixtures of C^{14} and C^{12}-glycine. After exposure they were shucked and the meat rinsed about 5 seconds in tap water, blotted, weighed and placed in 80% ethanol (ETOH). During a period of approximately 48 hours the ETOH extracts and tissue were agitated several times. Two 1 ml aliquots of each extract were analyzed for radioactivity with a Beckman 200-LS liquid scintillation counter and the C^{14}-activity expressed as counts per minute per mg ash-free weight (cpm/mg). Initial and final samples of media were also counted in all experiments.

In one instance a flowing system was utilized to expose *Rangia* to C^{14}-glycine over a 16-hour period. Water of 15‰ S was filtered through a 0.45 μ Millipore filter before the addition of glycine. A peristaltic pump (1 ml/min) was used to pull water through individual containers with *Rangia* and the effluent was collected in a fraction collector. The source medium and the chambers were constantly mixed by use of air-powered magnetic stirrers. Effluents and ETOH extracts of the animals were counted by liquid scintillation.

To obtain data on short term (less than one hour) uptake, the gills (demibranchs) were removed from acclimated animals and utilized in an uptake experiment. After dissection, gills were maintained in water of the appropriate salinity and then transferred after blotting to smaller containers with the exposure media. Groups of gills were removed from the C^{14}-glycine at intervals between 10 and 90 minutes, rinsed in three changes of water, and treated as described for whole animals.

A series of experiments were conducted to determine the kinetics of glycine uptake by *Rangia* at several salinities. When all animals were actively siphoning, a small volume of solution was added to each container, such that all clams were exposed to the same amount of C^{14}-glycine, but the total concentration of C^{14} plus C^{12} was between 10^{-3} and 10^{-5} molar glycine. After one hour of exposure in these concentration gradient studies, the usual extraction and counting procedures were conducted, and the velocity of uptake calculated as follows:

$$\frac{\text{cpm/g/hour (animals)}}{\text{cpm/ml (initial medium)}} \times \frac{\text{medium concentration}}{\text{(moles/ml)}} = \frac{\text{Velocity of Uptake}}{\text{(moles/g/hr)}}$$

These data were used to prepare a Lineweaver-Burke curve, where the reciprocal of the velocity is plotted against the reciprocal of the substrate concentration. As noted by other workers (Stephens, 1967), the use of this approach is not a suggestion that uptake is an enzyme-dependent process.

Glycine release

To examine the use of glycine in the osmoregulatory activity of the *Rangia*, animals were first exposed to C^{14}-glycine for four hours at a salinity of 22‰. After exposure, three clams were extracted as usual, while the remainder were placed in separate containers at salinities between 2 and 22‰ for 24 hours. Samples of medium from each container were taken at several time intervals, and the counts were expressed as radioactivity released in cpm/mg ash-free dry weight.

Fate of glycine

Animals which had been acclimated to various salinities were exposed to C^{14}-glycine for 2 hours and washed free of the radioactivity by six successive changes of the appropriate salinity water. They were then maintained in glycine-free

TABLE I.

Flow sheet for biochemical separation of the major tissue components

1. Extract 0.8 g wet weight of homogenized tissue in 10 ml of 80% ETOH for 24 hours.
2. Centrifuge* and pour off supernatant, repeat with 5 ml wash of 80% ETOH.
3. Extract lipids from pellet with 10 ml of ether-70% ETOH (3:1 v/v) for 18 hours with frequent mixing, centrifuge, repeat with 5 ml of ether-ETOH for 2 hours.

↓

Combined supernatants (lipids)
1. Evaporate in vacuum desiccator (insert a small beaker of paraffin oil in desiccator).
2. Weight when dry.

Pellet
1. Add 5 ml TCA (trichloroacetic acid) and mix well.
2. Heat to 80° C for 30 minutes, then hold at 50° C for 2 hours.
3. Cool and centrifuge, repeat with 5 ml of cold TCA.

↓

Combined supernatants (glycogen and nucleic acids)
1. Make the supernate 52% ETOH.
2. Allow to stand at 0° C for 18 hours.
3. Centrifuge.

Pellet (proteins)
1. Wash twice with 10 ml of absolute ETOH.
2. Dry to constant weight at 95° C.

↓

Supernatant (nucleic acids)
1. Remove TCA by extraction with 30 ml of ether in separatory funnel. Repeat 4 times.
2. Evaporate to dryness at 95° C.

Precipitate (glycogen)
1. Wash twice with 5 ml of absolute ETOH.
2. Dry to constant weight at 95° C.

* All centrifugation at 3000 rpm for ten minutes.

water of the appropriate salinity for periods up to 99 hours. At various intermediate intervals samples from each salinity were extracted in 80% ETOH as described above, and the remaining alcohol insoluble (incorporated) components were determined as follows: tissue was washed with alcohol, blotted and homogenized in a micro-Waring blender with enough 80% ETOH to bring the volume to 50 ml. Three 1 ml samples of each homogenate were solubilized with 1 ml of Protosol (New England Nuclear), neutralized with glacial acetic acid and counted by liquid scintillation. The alcohol soluble and insoluble radioactivities were corrected by use of quenching curves, and the data were expressed as per cent of

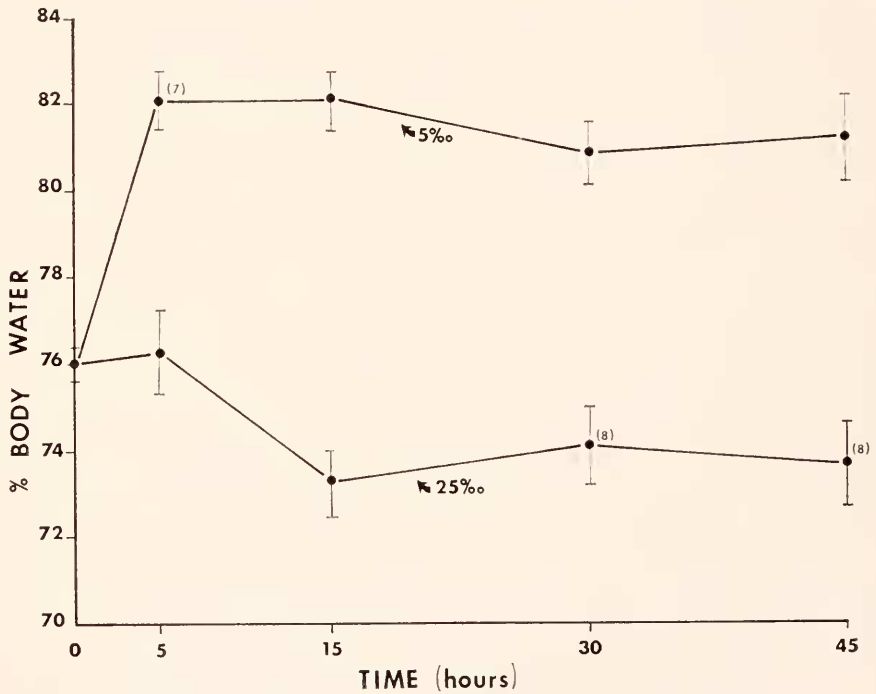

FIGURE 1. Volume regulation as a function of time. Vertical bars indicate standard deviations (SD), where n = 10 (except where noted).

the total activity. This method facilitates comparisons between salinities and compensates for individual differences in total activity.

Additional samples of the homogenates were quantitatively separated into protein, nucleic acids, lipids and polysaccharides (glycogen). The basic methodology (outlined in Table I) used was that of Shibko, Koivistoinen, Tratnyek, Newall, and Friedman (1967) with modifications as suggested by Graff (1970). After their qualitive separation, the four tissue fractions were prepared for counting as follows: first, the dried components were put into solution with 5 ml of 10% KOH, using heat where necessary. Then 1 ml of each tissue fraction was neutralized with glacial acetic acid and counted using the Beckman LS-200B

liquid scintillation system with an Aquasol cocktail. This uniform preparation for counting greatly reduced any differences in the quenching properties of the different fractions.

Results

Per cent body water and per cent ash

The percentage of tissue water increased by approximately 8 per cent (73 to 80.8%) as the acclimation salinity decreased from 32 to 1‰ S. The inorganic

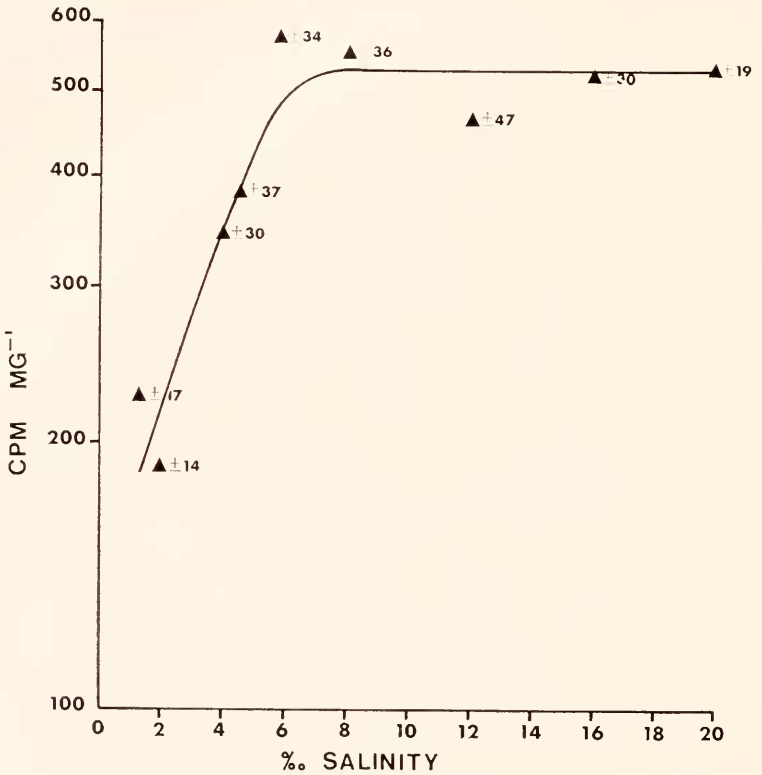

Figure 2. Ethanol soluble radioactivity of whole *Rangia* as a function of salinity; exposed to 1×10^{-5} molar glycine for 5 hours. SD shown as ±, where n = 6.

constituents of the tissue (ash) increased from 2.5% at 1‰ S to 7.6% at 32‰ S. The data exhibited good linear fit in both cases, with correlation coefficients of 0.964 and 0.995, respectively. In a few instances where it was not practical to determine ash-free dry weight directly, curves prepared from the above data were used to convert the values.

When specimens of *Rangia,* acclimated to 15‰ S, were immediately transferred to 5 or 25‰ S, the rate of change in per cent body water was measured (Fig. 1). It should be noted that those animals exposed to a decrease in salinity (5‰ S)

had already reached their approximately new steady state (82%) by the 5 hour interval. Clams transferred to 25‰ S presumably had not begun active siphoning by the 5 hour interval, since relatively little change had occurred in the per cent of body water. By the 15 hour interval both groups of clams had reached a level approximating their final steady state.

Uptake of glycine

Uptake of glycine by whole animals, as a function of salinity, is shown in Figure 2. The values plotted on the logarithmic scale represent the ethanol soluble activity after 5 hours of exposure in a 1×10^{-5} molar solution of $C^{14} + C^{12}$-

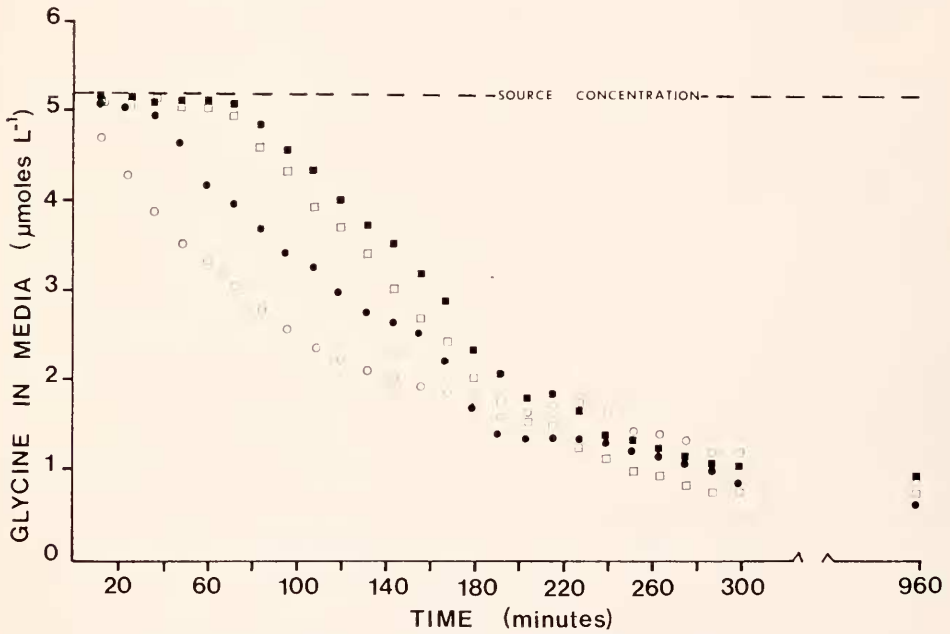

FIGURE 3. Glycine uptake by 4 individuals in separate flow-through chambers.

glycine (700 cpm = 1×10^{-3} micromoles). Resulting activities remained relatively constant (450–550 cpm/mg or about 0.7 μ moles/g) at salinities from 20 to 6‰ but decreased rapidly at lower salinities. The uptake at 6‰ S was shown to be 3 times that at 2‰ S. The uptake levels at the higher salinities (6–20‰ S) represent a 75 fold accumulation of glycine over that in the ambient medium.

The nature of the uptake process over time is shown in Figure 3. From these data it appears that the process is a continuous one, at least at the concentrations and time intervals tested. This experiment was conducted at 15‰ S with the glycine being introduced via a flow-through system. Uptake of glycine during the first 300 minutes was apparently dependent on the activity of each of the four clams, since the rate at which a constant differential was reached varied. From 300 minutes to 960 the removal of glycine was very stable. The 4.4 μ mole differential,

between source and outflow, presented on the graph represents a rate of glycine uptake equal to 0.264 μ moles per hour (calculated using the flow rate of 0.06 l/hr). Using the average ash-free dry weight of the clam tissue (1.58 g) the uptake was 0.161 μ moles/g/hr, for a period of approximately 11 hours. At a flow rate of 1 ml per minute there was considerable reduction in the concentration of dissolved oxygen present in the chambers after 16 hours of exposure. The concentrations were in the range of from 1 to 2 ml O_2 liter at termination and unpublished data indicate that respiration at these levels is approximately 0.5 ml O_2/g ash-free dry weight/hr. Since constant dissolved oxygen (D.O.) readings were not taken, it is not possible to relate D.O. to uptake, but the constant rate of glycine removal from

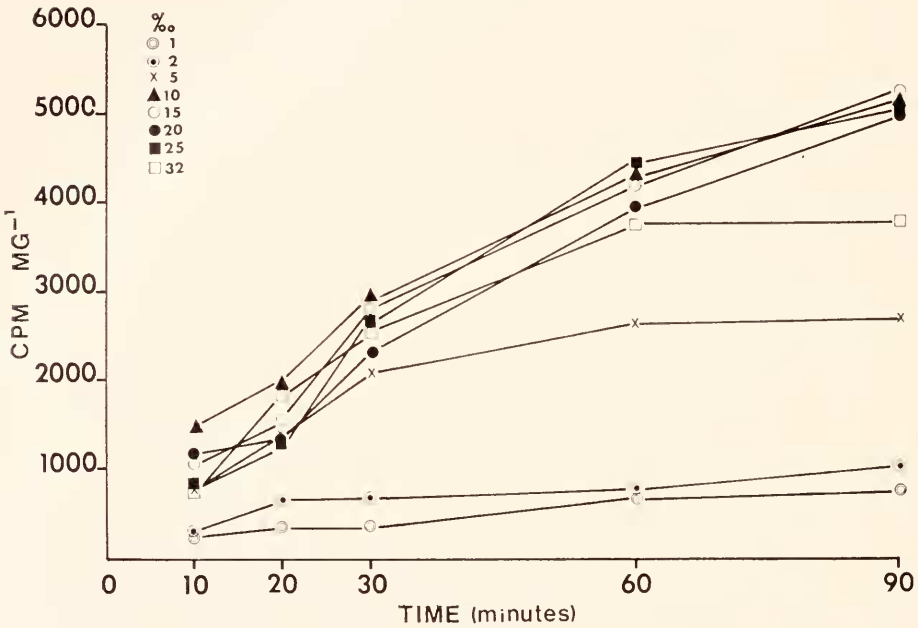

FIGURE 4. Ethanol soluble radioactivity of gill tissue as a function of salinity and time. SD values were approximately 7% of the plotted points, where n = 4.

3 to 16 hours, while D.O. was decreasing, indicates that uptake was independent of D.O. concentration.

The uptake by *Rangia* gills at various salinities from a 3.8×10^{-6} molar solution of C^{14} plus C^{12}-glycine is shown in Figure 4. These gills were removed immediately before exposure from clams that had been acclimated to each of the test salinities. Uptake was quite uniform and linear for gills at 10 through 25‰ S. The radioactivity associated with the gills at 1 and 2‰ S was relatively small and increased only slightly from 10 to 90 minutes. Uptake of glycine was intermediate for gills tested at 5 and 32‰ S, with the uptake rate sharply decreasing at 30 and 60 minutes, respectively. The data may be converted to μ moles of glycine by using a ratio of 1000 cpm/mg = 0.74 μ moles/g. After 90 minutes of exposure the gills at 10, 15, 20 and 25‰ had removed approximately 3.84 μ moles/g, while those at

1 and 2‰ had only accumulated 0.56 and 0.75 μ moles/g, respectively. The intermediate uptake at 5‰ S was expected from data on whole animals (Fig. 2), but the reduced uptake by gills at 32‰ after 90 minutes was not anticipated and will be discussed later. It should be noted that at no time was the glycine concentration in the exposure media reduced below 1×10^{-6} molar, and was therefore not limiting.

The relationship between the ambient glycine concentration and the rate of uptake by whole *Rangia* is shown in Table II. A Lineweaver-Burke plot of these data produced regression lines for each salinity with correlation coeffcients (Table II) which indicate excellent fit of the data to the curves. The standard estimate of error for each curve was less than 0.2, with the exception of one 2‰ S curve,

TABLE II

The relationship between the ambient glycine concentration (S) and the rate of uptake (V) at different salinities. Each V value represents the mean of 5 individuals. Kt represents that concentration of glycine at which uptake is half maximal (V_{max}).

Salinity (‰)	S ($\times 10^{-4}$ Molar)	V (μ moles/g/hr)	Slope of 1/V *versus* 1/S	V_{max} (μ moles/g/hr)	Kt ($\times 10^{-4}$ Molar)	Correlation coefficient
2	10.0	0.900	0.491	0.851	0.418	0.993
	5.0	1.200				
	1.0	0.457				
	0.5	0.445				
	0.1	0.166				
2	10.0	1.253	0.464	1.025	0.476	0.987
	5.0	0.819				
	1.0	0.633				
	0.5	0.545				
	0.1	0.178				
4	10.0	2.638	0.342	2.849	0.974	0.989
	5.0	1.905				
	1.0	1.478				
	0.5	1.078				
	0.1	0.264				
6	10.0	2.859	0.159	2.079	0.331	0.987
	5.0	2.398				
	1.0	1.289				
	0.5	1.054				
	0.1	0.492				
10	10.0	—	0.172	2.703	0.462	0.992
	5.0	2.839				
	1.0	1.468				
	0.5	1.608				
	0.1	0.478				
11	10.0	2.710	0.137	2.288	0.314	0.986
	5.0	2.263				
	1.0	1.772				
	0.5	1.171				
	0.1	0.561				

which was 0.4. When 95% confidence limits were prepared for each curve, the limits for 6, 10 and 11‰ S overlapped, while those for 4‰ were significantly different from the latter and also those enclosing both 2‰ S curves. Since the intercepts of the 4, 6, 10 and 11‰ S curves were nearly equal, the confidence limits did overlap as they approached the Y-axis (at higher concentrations). The slopes of the curves for 2 and 4‰ S are approximately the same, and those for 6, 10 and 11 are relatively close to each other. The data in Table II appear to represent three distinctive patterns of uptake. First, the data from the two separate experiments at 2‰ S are quite similar and indicate a slow rate of uptake, characterized by a low V_{max} (0.851–1.025). Secondly, the values for slope, V_{max} and Kt are relatively uniform for uptake of glycine at 6, 10 and 11‰ S. Finally, up-

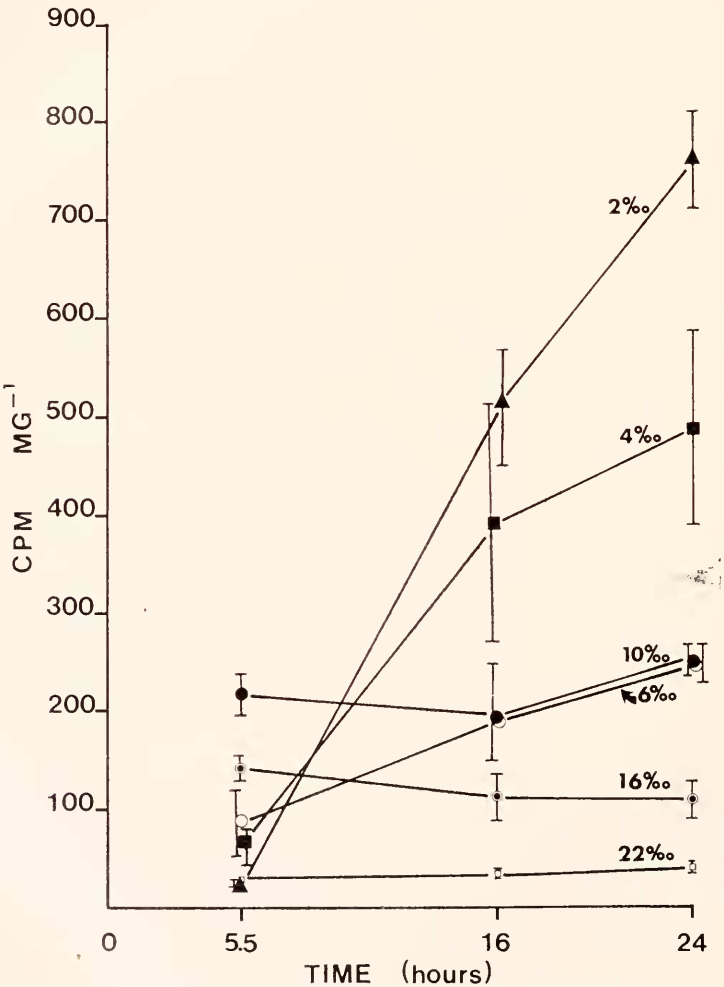

FIGURE 5. Release of C^{14}-activity as a function of salinity and time; *Rangia* previously exposed for 5 hours to C^{14}-glycine at 22‰ S. Vertical bars are standard errors, where n = 3.

take at 4‰ S appears to exhibit characteristics intermediate between the latter two groups, since the slope was high (as at 2‰ S) and the Kt was very large (0.974) with the resulting V_{max} similar to those of 6, 10 and 11‰ S.

When specimens of *Rangia* were first exposed to C^{14}-labeled glycine at 22‰ S and later moved to salinities of 16, 10, 6, 4 and 2‰, the accumulated radioactivity was released as shown in Figure 5. With decreasing salinity there was a corresponding increase in the radioactivity released into the media. The greatest release in radioactivity, which occurred after 24 hours, was at 2 and 4‰ S (762 and 440 cpm/mg, respectively). The release at 6‰ S was similar to that at 10‰ S (249 and 255 cpm/mg) and only slight amounts of radioactivity appeared at the higher salinity of 16‰. The 42 cpm/mg obtained at 22‰ was due to "Hot" exposure

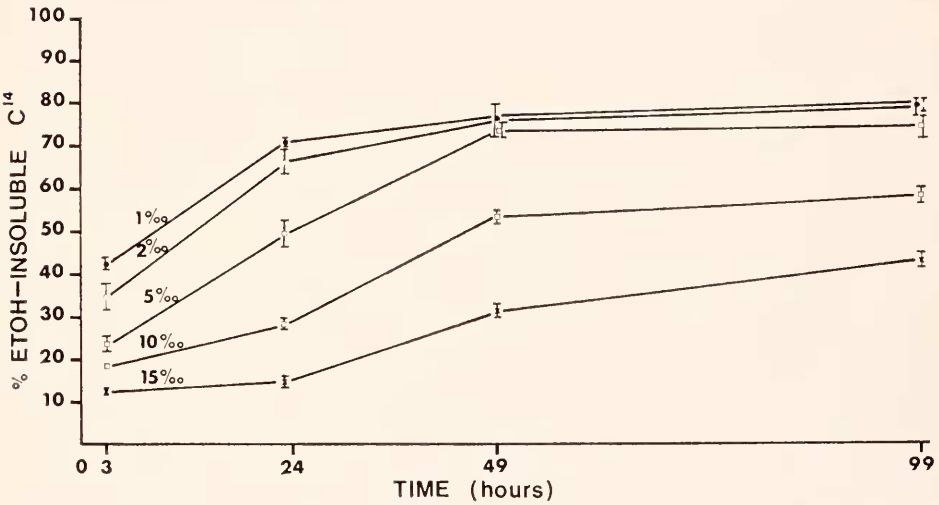

FIGURE 6. Percentage of total activity in the ETOH-insoluble fraction as a function of salinity and time. Vertical bars indicate SD, where n = 4.

media carried over when the clams were transferred to fresh media. The low levels of released activity at the 5.5 hour interval for the clams at 2 and 4‰ S was in all probability due to the extreme salinity shock inhibiting the siphoning activity of the clams.

Fate of glycine

The first aspect of a study to determine the fate of the glycine after uptake was to examine the percentage of activity that remains as free pool compounds and the percentage that was incorporated into the tissue components . These percentages are plotted versus time for salinities 1 through 15‰ S (Fig. 6). The components that are soluble in 80% ethanol have been defined as free pool components and the insoluble portion as incorporated compounds. Only the incorporated or ETOH insoluble fraction is plotted, as the pool or ETOH soluble fraction is the reciprocal of Figure 6. The most rapid and greatest incorporation occurred at the lower salinities of 1, 2, and 5‰ S, resulting in approximately 75% of the total activity

being converted to ETOH-insoluble components 49 hours after exposure to the labeled glycine. During the same time interval the clams in 10 and 15‰ S had only incorporated 54 and 32%, respectively, of the total accumulated glycine. At 99 hours the figures for the *Rangia* at 1, 2 and 5‰ S had not changed appreciably. However, incorporated radioactivity had increased for the animals at 10 and 15‰ S (58 and 44%, respectively). The total (free pool and incorporated) activity, for the 99 hour interval, is plotted against salinity in Figure 7. The total C^{14}-activity due to accumulation of labeled glycine was 3.5 times greater at 10 and 15‰ S than at 1‰ S. The total activity present in FAA pools and tissues of the animals de-

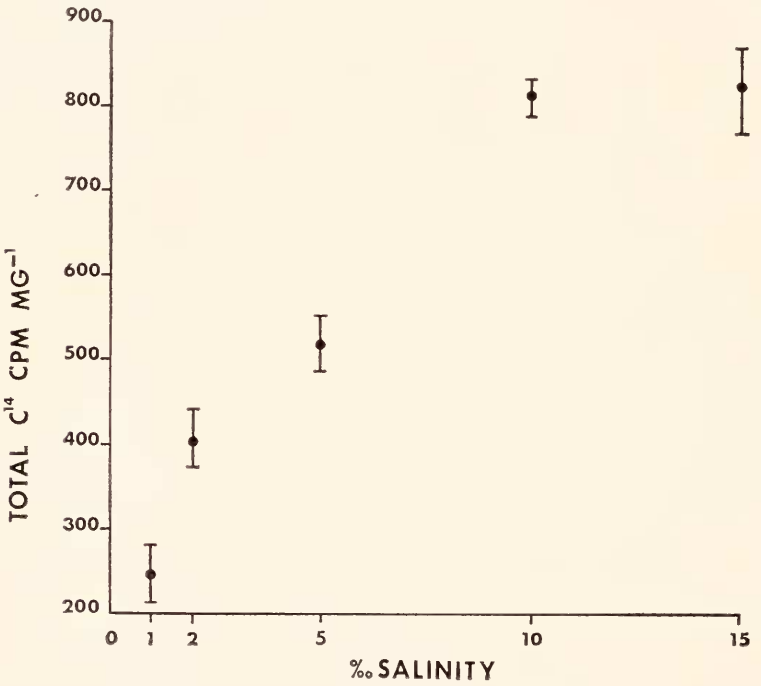

FIGURE 7. Total C^{14}-activity 99 hours after exposure to glycine at various salinities; vertical bars = SD, where n = 4.

creases sharply below 10‰ S. A comparison was made between the total activity present in the clams at 24 hours after exposure to that at 99 hours. The results of the comparison are presented in Table III as the per cent decrease in the total tissue activity and as the equivalent in micromoles of glycine per gram of tissue. It appears that with increasing salinity there was an increase in the utilization as well as uptake of glycine.

In Figures 8 and 9 the percentages of the incorporated activity which were found in the protein, nucleic acid, glycogen, and lipid fractions are presented for the 3 and 99 hour intervals, respectively, after exposure to C^{14}-labeled glycine. These fractions made up the following percentages by weight of the total tissue components: protein, 59.4 ± 4.9; nucleic acid, 15.8 ± 1.3; glycogen, 19.0 ± 5.3; and

lipid, 5.7 ± 1.8. The ± refers to the standard deviation, where n = 39. These percentages total very close to 100% which was expected, since the free pool compounds were extracted before analysis and the inorganic matter was subtracted from the total weight. There was no significant difference in the percentages of

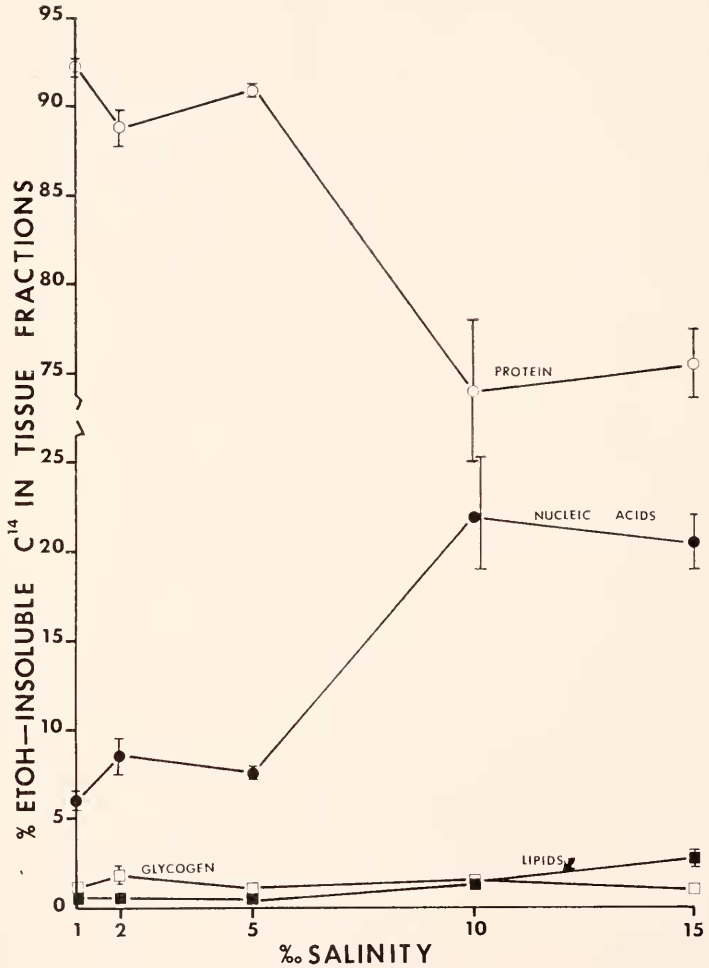

FIGURE 8. Percentage of ETOH-insoluble activity in the various tissue fractions 3 hours after exposure to C^{14}-glycine, vertical bars = SD, where n = 4.

the tissue components at the different salinities. There was, however, a significant difference in the fate of the labeled glycine with regard to both salinity and time. After three hours of exposure to C^{14}-glycine those clams at 1, 2 and 5‰ S had used approximately 90% of the incorporated glycine in protein synthesis, while the clams at 10 and 15‰ S had used only 75%. This difference was accounted for by a higher percentage of labeled nucleic acids in animals from the 10 and 15‰

salinities. After 99 hours the percentage of C^{14} in the protein of the low salinity clams had dropped to 87% and the percentage in the clams at 10 and 15‰ S had increased to 93%. The nucleic acid fractions at 10 and 15‰ S had decreased to a level where there was no significant difference with salinity at 99 hours. The percentage of C^{14} activity in the glycogen fractions had increased during the 96 hours from 1 or 2% to 5 or 6% at the low salinities. The C^{14}-activity associated with lipids increased slightly with salinity at the 3 hour interval (Fig. 8), but did not show any variation with salinity at the 99 hour period (Fig. 9). As pointed out in Table III the loss in the total tissue activity after 75 hours ranged from 9.2 to 69.2%, depending on salinity. This loss of activity was not accounted for by an increase in the media activity, thus the C^{14} must have been expelled as $C^{14}O_2$, indicating metabolism of labeled compounds.

FIGURE 9. Percentage of ETOH-insoluble activity in the various tissue fractions 99 hours after exposure to C^{14}-glycine; vertical bars = SD, where n = 4.

Discussion

When relating the physiological response of estuarine organisms to salinity it is extremely important to quantitate the data in a manner which is independent of salinity. Over the salinity range tested (1 to 32‰ S) the per cent body water was found to decrease from approximately 81 to 73%. Since clams acclimated to 32‰ S contain significantly higher levels of inorganic materials than those at 1‰ S, the use of dry weight is not as accurate as ash-free dry weight in the quantitation of metabolic processes. The inorganic constituents (ash) were shown to increase from 2.5% at 1‰ S to 7.6% at 32‰ S. The data for per cent body water and per cent ash compare quite closely to those of Allen (1961), with the exception that no decrease in the percentage dry weight or ash was noted at 25‰ in this study. All uptake data were calculated in terms of radioactivity or micromoles per unit ash-free dry weight.

As might be expected, the results of studies on the rate of volume regulation

Table III

The percentage decrease in radioactivity and μ moles of glycine in Rangia *tissue at various salinities. The decrease occurred between 24 and 99 hours after exposure*

Salinity (‰)	Per cent decrease in total radioactivity	Decrease in glycine (μ moles/g tissue)
1	9.2	0.015
2	21.3	0.067
5	45.5	0.260
10	53.8	0.419
15	69.2	1.108

compare closely with the earlier data on osmoregulation (Bedford and Anderson, 1972). In both instances, a new steady state was reached by the *Rangia* in approximately 24 hours, after transfer to media 10‰ hypo- or hyperosmotic to the acclimation medium. Again, the clams exhibited a more rapid rate of acclimation to the new environment under conditions of dilution (Bedford and Anderson, 1972). Inhibition of siphoning activity for at least 5 hours was apparent (Fig. 1) when animals were transfered from 15 to 25‰ S.

At the concentrations examined, *Rangia* was found to remove glycine from solution at all salinities tested. In the curves relating uptake to salinity there was a breaking point at approximately 6 to 10‰ S. At salinities of 6‰ or greater the uptake of glycine proceeded quite rapidly and at a rate which was independent of salinity. The single exception in the data was the suppression of uptake by gill tissue acclimated to 32‰ S. It should be noted that although specimens of *Rangia* survive well as adults in the laboratory at 32‰ S, they do not occur in habitats which attain salinities greater than 25‰ S for any significant amount of time. These clams as well as their larvae prefer salinities of 15‰ or less (Bedford and Anderson, unpublished data).

By various means, it has been shown that the uptake of glycine by whole *Rangia* and by their gill tissue declines sharply at salinities of 5‰ or less. After one hour of exposure to the same concentration of C^{14}-glycine, gill tissue from

clams acclimated to 10‰ S contained nearly an order of magnitude greater alcohol soluble radioactivity than gills from 1‰ S animals (Fig. 4). It should be noted that uptake of glycine by gill tissue occurred at a rate which was much more rapid and greater levels were obtained when compared to whole animals. Of course this was to be expected, since on a per unit weight basis gill tissue is certainly the most metabolically active tissue of bivalves. In addition to being the site for respiratory exchange, water propulsion, food sorting and ionic regulation, the gill has been shown to be responsible for a major portion of the glycine removal.

It would appear that some stress is exerted on the whole animals at salinities of 5‰ and less and also on the gill tissue at 32‰ S. As it has been shown (Bedford and Anderson, 1972) that *Rangia* maintain their blood at about 60 milliosmoles per liter above the environment at salinities below 5‰, presumably this osmotic work could be considered a stress. Stephens (1964) found that the uptake by *Nereis limnicola* decreased sharply at approximately the point at which chloride regulation began (14‰ S). He stated in a later publication (Stephens, 1967 page 371), "It may be that the processes that underlie osmotic regulation are incompatible with the rapid accumulation of amino acids from the ambient medium." Whether we consider the reduction of uptake at low salinity due to stress, competition for energy or competition for transport sites on the membrane is perhaps academic at this time. Much more information is required regarding transport mechanisms and their energy demands.

In addition to the possibility that the total osmotic concentration of the medium affects the uptake system, individual inorganic ions may influence the permeability and/or transport systems of the membrane. Although this subject has not been investigated at length, our unpublished results and those of Preston and Stephens (1969) and Stephens (1964) indicate that reductions in levels of sodium or chloride are not directly responsible for suppression of uptake at low salinity.

Stephens (1968) and Chien, Stephens and Healey (1972) have demonstrated long-term removal of amino acids by polychaetes at a constant rate of accumulation. The results of this investigation have shown that not only was the short-term accumulation of glycine by gill tissue relatively linear for 90 minutes at salinities between 10 and 25‰, but also long-term removal by whole animals at 15‰ was constant over a period of approximately 11 hours. Using a flowing system, the clams were found to remove 0.16 μ moles of glycine/g/hour when supplied with a concentration of 5.2 μ moles of glycine per liter (Fig. 3).

The release of C^{14}-labeled material by *Rangia*, which had previously been exposed to C^{14}-glycine and then transfered to various dilutions, was, as expected, dependent on the degree of dilution. Although no attempt was made to identify the material released, it was assumed to be glycine, since Stephens (1964) found the majority of alcohol soluble radioactivity of *Nereis* in the form of glycine, several hours after exposure. Since CO_2 was not driven off before counting and since certain polychaetes have been shown to rapidly interconvert amino acids in the FAA pool (Wong and Stephens, 1970), the character of the radioactivity remains uncertain. The use of free amino acids in the volume regulation and osmoregulation of lamellibranch mollusks is well documented (Virkar and Webb, 1970; Gilles, 1972; Allen, 1961). It is interesting to note that the release of radioactivity took place quite rapidly for *Rangia* subjected to slight reductions in salinity (6, 10 and

16‰), while those exposed to extreme dilution (2 and 4‰ S) had not released a significant amount until the 16 hour interval. To obtain better quantitative data relative to time, and to avoid the possibility of uptake of released material, such research should utilize a flow-through system.

From the results presented, it appears that the incorporation of the accumulated glycine by *Rangia* in response to salinity dilution follows much the same pattern as that of nereid polychaetes (Stephens, 1964). At all time intervals tested the per cent of ETOH insoluble C^{14}-activity (relative to the total activity) increased as the salinity decreased. In addition, the rate of incorporation, during at least the first 24 hours, increased as salinity decreased (Fig. 6). Further evidence of rapid incorporation of accumulated glycine by animals at low salinity is the high level of labeled protein in clams 3 hours after exposure (Fig. 8). It seems that nucleic acid synthesis was still the major activity of higher salinity *Rangia* at the 3 hour interval, but they had reached the same level of protein synthesis by the 99 hour analysis (Fig. 9). This type of response by organisms acclimated to low salinities would be a functional advantage, allowing them to rapidly transfer the glycine from the osmotically active FAA pool and yet utilize the compounds in synthetic pathways.

Although *Rangia* at salinities of 5‰ or less transferred the glycine from the FAA pool to protein and other large compounds at a rapid rate, the turnover rate of the total radioactivity was relatively slow. The total C^{14}-activity associated with the clams decreased over the 75 hour period at rates which increased as salinity increased (Table III). Apparently, *Rangia* maintained at salinities of 10‰ or greater utilize the accumulated glycine at a higher rate by conversion to secretory, excretory and respiratory products. It might be assumed that differences in the respiratory rate of the clams at various salinities would account for the variations in glycine utilization. However, a considerable amount of data (Bedford and Anderson, in preparation) demonstrate that the rate of oxygen consumption by *Rangia* at salinities between 1 and 32‰ is quite uniform after acclimation.

Finally, the aspect of nutrition, which has been discussed at length in several studies (Stephens, 1963; Stephens, 1967; Stephens, 1968; Johannes, Coward and Webb, 1969; Stephens, 1972) should be examined. At a glycine concentration of 4.6×10^{-5} moles/liter (Kt) the rate of uptake by whole animals was 101 μg of glycine/g/hr at 10‰. Since 2.2 mg of glycine requires roughly 1 ml of oxygen for complete oxidation (Stephens and Virkar, 1966), and since at 10‰ and 22° C the clams require 1.2 ml O_2/g ash-free dry weight/hr, the accululated glycine represents approximately 3.8% of the total oxygen requirement of *Rangia*. At 2‰ S the glycine taken up was equivalent to only 1.5% of the energy necessary for respiration. The concentration of 4.6×10^{-5} is perhaps high for a single amino acid even in an estuary, but Stephens (1963) has reported values between 6×10^{-5} and 1×10^{-4} moles/liter for total free amino acids in interstitial water of mud flats. Even if one assumes higher levels of glycine are present in the habitat of *Rangia* or that they are simultaneously removing other amino acids, it would appear that these mollusks can not support as large a portion of their metabolic energy needs as other invertebrates investigated. Ecological studies (Darnell, 1958) have shown that in addition to feeding on phytoplankton, *Rangia* utilize detritus, which is generally in abundance in the upper reaches of the estuary.

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Summary

1. The effect of salinity on the uptake of dissolved C^{14}-labeled glycine by *Rangia cuneata* and isolated gill tissue was studied.

2. The percentage water in *Rangia* tissue ranged from 81% at 1‰ S to 73% at 32‰ S, while the ash-free dry weights at these same salinities were 2.5% and 7.6%, respectively. The per cent water and per cent ash values were linear throughout this salinity range.

3. The uptake of glycine was relatively uniform at salinities between 6 and 32‰ for whole animals and between 10 and 25‰ for gill tissue.

4. At salinities below 6‰ uptake decreased sharply and in a linear fashion to a low at 1‰ S. Suppression of uptake was also exhibited when gill tissue was exposed for 90 minutes at 32‰ S.

5. With increasing salinity the percentage utilization of accumulated glycine increased, but the rate of glycine incorporation into the alcohol insoluble fraction decreased.

6. Removal of glycine by gill tissue from salinities of 10 to 25‰ occurred at a relatively linear rate up to 90 minutes and the levels obtained were considerably higher than those for whole animals when compared on a basis of cpm per mg ash-free dry weight.

7. In a constantly flowing system *Rangia* at 15‰ S removed 0.16 μ moles of glycine/g/hour for a period of 11 hours, when supplied with 5.2 μ moles of glycine per liter.

8. The significance of the relationship between salinity and glycine uptake, and possible explanations for this relationship are discussed.

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